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A novel approach to childhood obesity: circulating chemokines and growth factors as biomarkers of insulin resistance

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Summary

Background: Insulin resistance (IR) in children with obesity constitutes a risk factor that should be precisely diagnosed to prevent further comorbidities.

Objective: Chemokines were evaluated to identify novel predictors of IR with clinical application.

Methods: We analysed the levels of cytokines (tumour necrosis factor [TNF] α and interleukins [ILs] 1β, 4, 6 and 10), chemokines (stromal cell derived factor 1α, monocyte chemoattract protein [MCP] 1, eotaxin and fractalkine) and growth factors (brain-derived neurotrophic factor, pro-fibrotic platelet-derived growth factor [PDGF-BB] and insulin-like growth factor 1) in serum of prepubertal children with obesity (61 girls/59 boys, 50% IR and 50% non-IR) and 32 controls. Factor analysis, correlation, binary logistic regression and receiver operating characteristic analysis of combined biomarkers were used to validate their capability for preventive interventions of IR.

Results: Changes in MCP1, eotaxin, IL1β and PDGF-BB were observed in IR children with obesity. Bivariate correlation between stromal cell derived factor 1α, MCP1, eotaxin, TNFα, brain-derived neurotrophic factor and/or PDGF-BB explained the high variance (65.9%) defined by three components related to inflammation and growth that contribute towards IR. The combination of leptin, triglyceride/high-density lipoprotein, insulin-like growth factor 1, TNFα, MCP1 and PDGF-BB showed a sensitivity and specificity of 93.2% for the identification of IR. The percentage of correct predictions was 89.6.

Conclusions: Combined set of cytokines, adipokines and chemokines constitutes a model that predicts IR, suggesting a potential application in clinical practice as biomarkers to identify children with obesity and hyperinsulinaemia.

Keywords: ~~Chemokine~~, children, cytokine, insulin resistance, obesity.

Abbreviations: AUC, area under the curve; BDNF, brain-derived neurotrophic factor; HMW, high molecular weight; IGF, insulin-like growth factor; IL, interleukin; IR, insulin resistance; MCP, monocyte chemoattract protein; PDGF-BB, pro-fibrotic platelet-derived growth factor; ROC, receiver operating characteristic; SDF, stromal cell derived factor; T2DM, type 2 diabetes mellitus; TNF, tumour necrosis factor; TSH, thyroid-stimulating hormone

Introduction

Metabolic complications associated with obesity starting in childhood increase the risk of diseases such as type 2 diabetes mellitus (T2DM), dyslipidaemia and early cardiovascular disease in adulthood. Insulin resistance (IR) has been largely postulated as the onset of metabolic impairment underlying obesity and metabolic syndrome (1). However, diagnosis of IR in the paediatric population with obesity is still a matter of debate as the proposed indexes and diagnostic criteria for IR have been developed for adults and their application to children has not been specifically verified. Indeed, processes such as growth and puberty

affect insulin secretion and modulate tissue sensitivity to insulin's action (2). In the clinic, IR is usually estimated by using the homeostasis model assessment (HOMA) index and oral glucose tolerance test (OGTT) to determine glycaemia and/or insulinaemia in both fasting and post-ingestion states.

Insulin resistance and obesity in children are associated with low-grade systemic inflammation that is characterized by up-regulated production of pro-inflammatory cytokines (e.g. tumour necrosis factor [TNF] α, interleukin [IL] 6 and resistin) and immune system activation (3,4). Specifically, pro-inflammatory adipokines are induced to recruit immune system cells in response to tissue inflammation associated with obesity and IR (5,6). Monocyte and macrophage infiltration and high levels of pro-inflammatory chemokines, such as monocyte chemoattract protein (MCP) 1 and stromal cell derived factor (SDF) 1α, have been found in adipose tissue of obese and/or insulin-resistant mice and adult humans (7–9). Most chemokines, including eotaxin and fractalkine, are found in pre-adipocytes/adipocytes and show a differential response to growth and pro-inflammatory factors (10). In addition, fractalkine has been described as a potent modulator of insulin secretion and a predictor of IR and metabolic syndrome (11,12).

Although the relationship between brain-derived neurotrophic factor (BDNF) and IR or cardiovascular risk has not been described in children, extremely overweight children have been reported to exhibit low levels of BDNF, suggesting an inverse correlation between BDNF and leptin (13,14). In adults with obesity, metabolic syndrome has been associated with a decrease in pro-fibrotic platelet-derived growth factor (PDGF-BB) (15), with actions linked to insulin sensitivity through hepatic vascular permeability (16).

Because potential circulating biomarkers could ease the transition to routine use in clinical practice, the present study aimed to evaluate the contribution of a set of selected pro-inflammatory/anti-inflammatory cytokines (TNFα, IL1β, IL4, IL6 and IL10), chemokines (SDF-1α, MCP1, eotaxin and fractalkine) and growth factors (BDNF, PDGF-BB and insulin-like growth factor [IGF] 1) in obesity-associated hyperinsulinaemia in a large cohort of prepubertal children. We employed a statistical strategy to identify and validate the capability of these circulating biomarkers for prediction and diagnosis of IR in children with obesity, as well as to better understand its underlying pathophysiological mechanism.

Methods

Ethics statement

The study and protocols for recruitment were approved by the Scientific and Ethics Committee of the Hospital Infantil Universitario Niño Jesús de Madrid (17,18) in accordance with the 'Ethical Principles for Medical Research Involving Human Subjects' adopted in the Declaration of Helsinki of the World Medical Association (64th WMA General Assembly, Fortaleza, Brazil, October 2013), Recommendation No. R [97] 5 of the Committee of Ministers to Member States on the Protection of Medical Data (1997), and Spanish data protection act (*Ley Orgánica 15/1999 de Protección de Datos*). Written informed consent was obtained from all patients or their legal guardian after a complete description of the study.

Subjects

One hundred and twenty prepubertal (Tanner stage I) subjects with obesity (body mass index [BMI] > +2 standard deviation score according to Spanish standards and International Obesity Task Force references for children (19,20); 61 girls/59 boys, 50% IR and 50% non-IR in each group) were studied (18). A control group of 32 healthy children (17 girls/15 boys, BMI −1.5 to +1.5 standard deviation score) was also included in the present study. The anthropometric and metabolic characteristics of the control group and group with obesity (IR and non-IR subgroups) were evaluated.

Recruitment

Patients consulted the Department of Endocrinology for being overweight. They were studied to rule-out any underlying pathological condition before enrolment. BMI was recorded and standardized (BMI Z-score). An OGTT (1.75 g of glucose/kg; maximum 75 g) was performed after 12-h overnight fasting, and blood samples were drawn at 0, 30, 60 and 120 min for glucose and insulin measurements. Fasting venous blood samples were collected and centrifuged at 4°C. Serum was separated and frozen at −80°C until assayed. Serum samples were used to quantify biochemical parameters (glucose, uric acid, total cholesterol, high-density lipoprotein [HDL] cholesterol, low-density lipoprotein [LDL] cholesterol and triglycerides [TGs]), glycosylated haemoglobin, hepatic transaminases (glutamate-oxaloacetate transaminase, glutamate-pyruvate transaminase and gamma-glutamyl transferase) and

high-sensitivity C-reactive protein, as well as hormonal (insulin, IGF-binding protein [BP] 3, leptin and adiponectin), cytokine (TNFα, IL1β, IL4, IL6 and IL10), chemokine (SDF-1α, MCP1, eotaxin and fractalkine) and growth factors (BDNF, IGF-1 and PDGF-BB).

Patients were considered IR if they had fasting insulin >15 μU mL^{-1} plus one or more of the following criteria during the OGTT: peak insulin at 30–60 min > 150 μU mL^{-1} or insulin at 120 min > 75 μU mL^{-1} after oral glucose overload (2). The area under the curve (AUC) for insulin and glucose, the HOMA-estimated IR (HOMA-IR) index and the whole-body insulin sensitivity index were calculated as previously reported (17).

ELISAs and chemiluminescent immunoassays

ELISA kits were used to measure serum concentrations of insulin, leptin and total and high molecular weight (HMW) adiponectin (Linco Research, St. Charles, MO, USA) with the sensitivities and assay variations for these analyses being previously reported (21). Serum IGF-1 and IGFBP-3 levels were measured with automated chemiluminescent immunoassays (Immunodiagnostic Systems, Tyne & Wear, UK), and free thyroxine and thyroid-stimulating hormone (TSH) were determined in the central clinical biochemistry laboratory of the hospital by chemiluminescent immunoassays from Beckman Coulter (Brea, CA, USA). All assays were performed according to the manufacturers' instructions.

Multiplexed bead immunoassay

Serum concentrations of BDNF, PDGF-BB, MCP1, SDF-1α, eotaxin, fractalkine, IL1β, IL4, IL6, IL10 and TNFα were measured by two multiplexed bead immunoassay kits from Affymetrix (Vienna, Austria) as previously described (22), with minor modifications (4,23). Sensitivity was approximately 0.5–4.7 pg mL^{-1}, mean intra-assay variation was 6.8% and mean inter-assay variation was 11.7% for all analyses.

Statistical analysis

Data are expressed as the *n* (%) and the mean ± standard deviation. Data were analysed using IBM SPSS software 23.0 (SPSS Inc., Chicago, IL, USA). Q16 Statistical data analysis was performed in four steps. First, the comparison of the quantitative variables (anthropometric and metabolic characteristics, and cytokine, chemokine and growth factor concentrations) between control group (n = 32) and group with obesity (n = 120) was performed using chi-squared test and Student's t-test as variables fit a normal distribution (Kolmogorov–Smirnoff test). Second, two-way analysis of variance (sex and IR as factors) and Bonferroni's *post hoc* analysis for multiple comparisons were carried out in the group with obesity (n = 120). Third, exploratory factor analysis with varimax rotation and bivariate relationships (correlation) between the variables selected was undertaken with the data from the group with obesity (n = 120) to determine the factors (components) that account for IR. Only variables with a factor loading of at least 0.3 (sharing at least 10% of the variance with a factor) were used for interpretation. Fourth, the backward method for binary logistic regression and receiver operating characteristic (ROC) curve analysis was used to determine the risk of IR. The steps were to obtain all possible combinations of the explanatory variables and to calculate the highest ROC–AUC of the resulting model with that combination (the model with the greatest discrimination power). Statistical significance was defined as $p < 0.05$.

Results

Anthropometric and metabolic characteristics in prepubertal subjects with obesity

Patients with obesity present higher serum levels of BMI Z-score, basal insulin, LDL cholesterol and TGs, an elevated HOMA-IR index and an increased ratio of TGs/HDL cholesterol and total cholesterol/HDL cholesterol (Table 1). Serum HDL cholesterol levels were lower in children with obesity than in control children. No differences were found regarding the remaining anthropometric variables (Table 1). T1

Anthropometric and metabolic characteristics in prepubertal patients with obesity and insulin resistance

Subjects with obesity were divided into IR (n = 60) and non-IR (n = 60), and each group was divided into boys (IR: n = 29; non-IR: n = 30) and girls (IR: n = 31; non-IR: n = 30). A sex-specific effect was found on age and waist circumference, circulating levels of fasting glucose and free thyroxine and the ratios of LDL cholesterol/HDL cholesterol and HMW adiponectin/adiponectin ($^{a/aa/aaa}p < 0.05/0.01/0.001$; Table 2). There was also an association T2

Table 1 Anthropometric and metabolic characteristics of prepubertal patients with obesity

Variables		Control (n = 32)	Obesity (n = 120)	p-value
Sex, n (%)	Girls	17 (53.12)	61 (50.83)	0.421[1]
	Boys	15 (46.87)	59 (49.16)	
Age, mean (SD)	Years	8.27 (2.18)	7.86 (2.40)	0.336[2]
BMI Z-score, mean (SD)		−0.29 (0.92)	5.19 (2.05)	**0.029**[2]
Basal insulin, mean (SD)	pmol L^{-1}	5.74 (2.85)	15.04 (8.85)	**0.002**[2]
Basal glucose, mean (SD)	mg dL^{-1}	89.06 (6.22)	92.23 (7.15)	0.469[2]
HOMA-IR, mean (SD)	Index	1.27 (0.68)	3.45 (2.01)	<**0.001**[2]
Uric acid, mean (SD)	mg dL^{-1}	3.86 (0.82)	4.56 (1.02)	0.542[2]
Total cholesterol, mean (SD)	mg dL^{-1}	168.96 (30.50)	161.08 (31.63)	0.775[2]
HDL cholesterol, mean (SD)	mg dL^{-1}	101.88 (24.11)	46.83 (12.58)	<**0.001**[2]
LDL cholesterol, mean (SD)	mg dL^{-1}	59.94 (15.51)	98.50 (26.51)	**0.015**[2]
Triglycerides, mean (SD)	mg dL^{-1}	39.12 (13.36)	78.04 (45.14)	**0.002**[2]
Triglycerides/HDL cholesterol, mean (SD)	Ratio	0.69 (0.34)	1.85 (1.51)	**0.008**[2]
Total cholesterol/HDL cholesterol, mean (SD)	Ratio	2.96 (0.65)	3.60 (0.89)	**0.022**[2]
LDL cholesterol/HDL cholesterol, mean (SD)	Ratio	1.80 (0.60)	2.23 (0.75)	0.103[2]
Free T4, mean (SD)	ng dL^{-1}	0.97 (0.13)	0.95 (0.96)	0.053[2]
TSH, mean (SD)	μU mL^{-1}	2.54 (1.39)	2.88 (1.45)	0.720[2]
Free T4/TSH, mean (SD)	Ratio	0.51 (0.30)	0.42 (0.30)	0.188[2]
IGFBP-3, mean (SD)	mg L^{-1}	4.02 (0.74)	4.44 (0.94)	0.265[2]

[1]p-value from chi-squared test.
[2]p-value from Student's t-test.
Bold numbers denote significant differences ($p < 0.05$).
BMI, body mass index; HDL, high-density lipoprotein; HOMA, homeostasis model assessment; IGFBP, IGF-binding protein; IR, insulin resistance; LDL, low-density lipoprotein; SD, standard deviation; T4, free thyroxine; TSH, thyroid-stimulating hormone.

between IR and age, waist circumference, insulin and glucose (fasting, 30, 60 and 120 min in OGTT), the percentage of glycosylated haemoglobin, HOMA-IR, whole-body insulin sensitivity index, AUC glucose, AUC insulin, leptin, adiponectin, HMW adiponectin, leptin/adiponectin ratio, uric acid, glutamate-pyruvate transaminase, gamma-glutamyl transferase, TGs, TGs/HDL cholesterol ratio, total cholesterol/HDL cholesterol ratio and TSH ($^{b/bb/bbb}p < 0.05/0.01/0.001$; Table 2). Interaction between factors (sex and IR) was only observed in the circulating levels of TSH ($^{c}p < 0.05$; Table 2).

Circulating concentrations of cytokines, chemokine and growth factors in prepubertal subjects with obesity

Patients with obesity had an increase in circulating levels of TNFα ($p < 0.001$), MCP1 ($p < 0.001$) and IGF-1 ($p < 0.05$) compared with the control group (Fig. 1a,e,h). On the contrary, patients with obesity had decreased concentrations of PDGF-BB compared with the control group ($p < 0.001$; Fig. 1f). No differences were found in the remaining factors (Fig. 1b–d,g and Table S1).

Circulating concentrations of cytokines, chemokine and growth factors in prepubertal patients with obesity and insulin resistance

We found a sex effect on PDGF-BB concentrations in serum ($F_{1,116} = 26.16$, $p < 0.01$). We also found an IR association with the circulating levels of IL1β ($F_{1,116} = 19.47$, $p < 0.001$), eotaxin ($F_{1,116} = 10.57$, $p < 0.01$), MCP1 ($F_{1,116} = 29.95$, $p < 0.001$) and PDGF-BB ($F_{1,116} = 21.64$, $p < 0.001$). Interactions between sex and IR were detected on the serum levels of IL1β ($F_{1,116} = 5.89$, $p < 0.05$) and IL4 ($F_{1,116} = 13.90$, $p < 0.001$). No significant effects or interaction was detected in the remaining factors (Fig. 1i–p and Table S2).

In both groups (girls and boys) with obesity, IR was associated with decreased serum levels of MCP1 compared with those of the respective female and male non-IR groups ($^{**}/^{***}p < 0.01/0.001$, respectively; Fig. 1m). Non-IR and IR boys with obesity had increased serum levels of PDGF-BB compared with the respective non-IR and IR girls with obesity ($^{\#/\#\#\#}p < 0.05/0.001$, respectively; Fig. 1o). Moreover, IR boys with obesity had increased serum levels of

Table 2 Anthropometric and metabolic characteristics of prepubertal patients with obesity regarding insulin resistance and sex effects

Variables, mean (SD)		Obesity non-IR (n = 60)		Obesity IR (n = 60)		Two-way ANOVA
		Girls (n = 30)	Boys (n = 30)	Girls (n = 31)	Boys (n = 29)	
Age	Years	6.03 (2.48)	7.89 (2.37)	9.07 (1.93)	8.47 (1.67)	aa/bbb
BMI Z-score		4.93 (1.33)	5.60 (2.28)	4.78 (1.46)	5.46 (2.70)	
Waist circumference	cm	75.04 (9.75)	83.79 (11.69)	85.17 (7.31)	90.15 (10.41)	aaa/bbb
Fasting insulin	pmol L^{-1}	8.46 (3.19)	9.65 (2.51)	21.62 (10.70)	20.39 (6.02)	bbb
Insulin 30 min	pmol L^{-1}	63.53 (26.60)	70.57 (25.67)	200.48 (95.63)	183.08 (86.87)	bbb
Insulin 60 min	pmol L^{-1}	48.68 (30.19)	63.84 (31.02)	173.82 (82.62)	156.60 (74.44)	bbb
Insulin 120 min	pmol L^{-1}	46.98 (16.12)	42.58 (17.18)	159.27 (75.60)	148.31 (63.91)	bbb
Fasting glucose	mg dL^{-1}	87.80 (5.18)	91.47 (5.92)	93.58 (5.75)	96.17 (8.68)	aa/bbb
Glucose 30 min	mg dL^{-1}	134.00 (19.87)	138.24 (21.27)	148.11 (23.88)	154.46 (23.15)	bbb
Glucose 60 min	mg dL^{-1}	118.16 (24.86)	122.93 (23.42)	138.66 (32.99)	137.82 (26.77)	bbb
Glucose 120 min	mg dL^{-1}	112.68 (12.62)	110.03 (11.05)	125.72 (22.07)	129.93 (22.65)	bbb
HbA1c	%	5.32 (0.34)	5.27 (0.29)	5.53 (0.38)	5.43 (0.45)	bb
HOMA-IR	Index	1.84 (0.70)	2.19 (0.61)	4.96 (2.18)	4.83 (1.46)	bbb
WBISI		5.10 (1.96)	4.23 (1.49)	1.63 (0.64)	1.56 (0.54)	bbb
AUC glucose		234.27 (29.14)	238.81 (27.62)	262.54 (40.23)	269.61 (36.21)	bbb
AUC insulin		94.33 (36.04)	107.66 (35.93)	316.43 (112.18)	284.82 (106.31)	bbb
Leptin	ng mL^{-1}	22.32 (8.92)	24.76 (13.13)	33.46 (11.00)	31.59 (13.19)	bbb
Adiponectin	ng mL^{-1}	15.28 (5.71)	17.17 (8.57)	13.54 (5.77)	13.10 (8.56)	b
hs-CRP	mg L^{-1}	1.89 (4.51)	2.12 (4.69)	2.05 (1.90)	2.12 (2.32)	
HMW adiponectin	ng mL^{-1}	6.22 (3.29)	6.76 (4.16)	5.42 (4.15)	4.07 (3.03)	b
Leptin/adiponectin	Ratio	1.70 (1.17)	1.79 (1.38)	2.85 (1.36)	3.25 (2.25)	bbb
HMW adiponectin/adiponectin	Ratio	0.40 (0.10)	0.37 (0.09)	0.39 (0.16)	0.32 (0.12)	a
Uric acid	mg dL^{-1}	4.39 (0.78)	4.35 (1.01)	4.57 (0.77)	4.93 (1.34)	b
GOT	U L^{-1}	31.03 (4.45)	29.93 (6.65)	28.80 (10.21)	30.41 (8.41)	
GPT	U L^{-1}	22.07 (7.82)	22.93 (8.27)	26.90 (19.70)	31.17 (17.55)	bbb
γGT	U L^{-1}	12.07 (3.34)	12.03 (4.71)	15.50 (4.52)	18.17 (6.12)	bbb
Total cholesterol	mg dL^{-1}	156.83 (27.49)	157.52 (32.26)	165.77 (30.66)	164.21 (35.93)	
HDL cholesterol	mg dL^{-1}	46.40 (13.96)	51.22 (12.46)	43.64 (10.08)	46.23 (12.58)	
LDL cholesterol	mg dL^{-1}	98.05 (22.64)	93.91 (27.81)	103.75 (26.83)	98.14 (28.56)	
Triglycerides	mg dL^{-1}	62.87 (26.91)	58.97 (26.72)	91.70 (39.06)	98.69 (64.13)	bbb
Triglycerides/HDL cholesterol	Ratio	1.47 (0.74)	1.23 (0.70)	2.24 (1.11)	2.47 (2.42)	bbb
Total cholesterol/HDL cholesterol	Ratio	3.55 (0.82)	3.19 (0.76)	3.94 (0.94)	3.69 (0.90)	bb
LDL cholesterol/HDL cholesterol	Ratio	2.27 (0.75)	1.94 (0.68)	2.49 (0.83)	2.22 (0.65)	a
Free T4	ng dL^{-1}	0.94 (0.07)	0.98 (0.12)	0.92 (0.07)	0.96 (0.11)	a
TSH	μU mL^{-1}	2.33 (0.96)	2.92 (1.87)	3.44 (1.33)	2.83 (1.29)	b/c
Free T4/TSH	Ratio	0.46 (0.16)	0.43 (0.20)	0.41 (0.51)	0.41 (0.19)	
IGFBP-3	mg L^{-1}	4.21 (0.95)	4.38 (0.96)	4.35 (0.66)	4.83 (1.09)	

'a' indicates two-way ANOVA sex effect. $^{a/aa/aaa}p < 0.05/0.01/0.001$.
'b' indicates two-way ANOVA insulin resistance effect. $^{b/bb/bbb}p < 0.05/0.01/0.001$.
'c' indicates two-way ANOVA interaction between sex and insulin resistance. $^{c}p < 0.05$.
ANOVA, analysis of variance; AUC, area under the curve; BMI, body mass index; GOT, glutamate-oxaloacetate transaminase; GPT, glutamate-pyruvate transaminase; γGT, gamma-glutamyl transferase; HbA1c, glycosylated haemoglobin; HDL, high-density lipoprotein; HOMA, homeostasis model assessment; HMW, high molecular weight; hs-CRP, high-sensitivity C-reactive protein; IGFBP, IGF-binding protein; IR, insulin resistance; LDL, low-density lipoprotein; SD, standard deviation; T4, free thyroxine; TSH, thyroid-stimulating hormone; WBISI, whole-body insulin sensitivity index.

PDGF-BB compared with those non-IR boys ($^{***}p < 0.001$; Fig. 1o). No significant differences between groups were observed in the remaining molecules analysed (Fig. 1i–l,n,p).

Non-IR boys and IR girls with obesity had lower serum levels of IL1β compared with non-IR girls with obesity ($^{*/***}p < 0.05/0.001$, respectively; Table S2). IR boys with obesity had lower circulating levels of

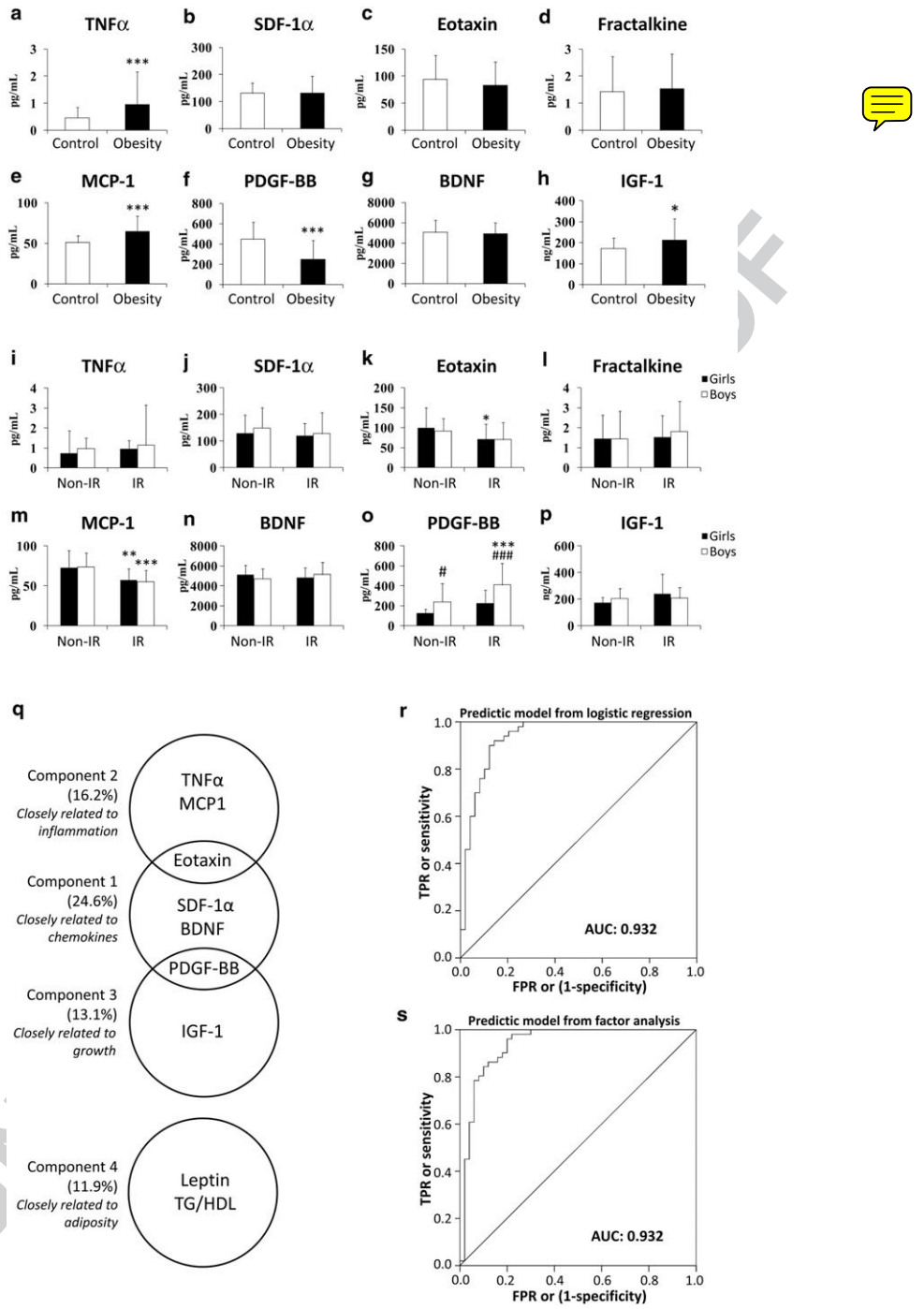

Figure 1 Circulating concentrations of tumour necrosis factor (TNF) α, stromal cell derived factor (SDF) 1α, eotaxin, fractalkine, monocyte chemoattract protein (MCP) 1, pro-fibrotic platelet-derived growth factor (PDGF-BB), brain-derived neurotrophic factor (BDNF) and insulin-like growth factor (IGF) 1 in (a–h) prepubertal patients with obesity compared with age-matched, sex-matched and BMI-matched healthy children (control) and (i–p) girls and boys with obesity and insulin resistance (IR) compared with those obese girls and boys without IR (non-IR). a–h: Student's *t*-test, $^{*/***}p < 0.05/0.001$ vs. control. i–p: Bonferroni's *post hoc* test, $^{**/***}p < 0.01/0.001$ vs. non-IR (girls or boys); $^{\#/\#\#\#}p < 0.05/0.001$ vs. girls (non-IR or IR). A diagrammatic representation of the factor analysis performed in 120 patients with obesity (q). Receiver operating characteristic (ROC) analyses of two predictive models from logistic regression (r) and factor analysis (s) were also represented. Logistic regression model: leptin, triglyceride/high-density lipoprotein (TG/HDL), IGF-1, SDF-1α, TNFα, MCP1, BDNF and PDGF-BB (ROC–AUC: 0.932). Factor analysis model: leptin, TG/HDL, IGF-1, TNFα, MCP1 and PDGF-BB (ROC–AUC: 0.932).

IL4 compared with both non-IR boys and IR girls with obesity ($^{\#\#}/^{\$\$}p < 0.01$, respectively).

Chemokines and growth factors as contributors to insulin resistance in prepubertal patients with obesity

After assessing factor analysis and correlations (n = 120; Table S3), the model selected contained nine independent variables: leptin, TG/HDL, IGF-1, SDF-1α, eotaxin, TNFα, MCP1, BDNF and PDGF-BB. We observed significant correlations between (i) eotaxin and SDF-1α (p = 0.006); (ii) MCP1 and SDF-1α (p = 0.003), eotaxin (p = 0.006) and TNFα ($p < 0.001$); (iii) BDNF and SDF-1α (p = 0.022) and eotaxin (p = 0.042); and (iv) PDGF-BB and SDF-1α (p = 0.02), eotaxin (p = 0.012) and BDNF (p = 0.025). The variances in the nine variables chosen (IGF-1 = 84%, TNFα = 78%, MCP1 = 77%, TG/HDL = 66%, PDGF-BB = 64%, leptin = 62%, eotaxin = 57%, SDF-1α = 56% and BDNF = 48%) are accounted for by four extracted components.

Four components (factors) together explained 65.8% of the variance that contributes towards IR (Fig. 1q and Table S4). The most influential factor (component 1) explained 24.64% of the total variance and can be interpreted as mitogenic signalling related to an immune response as SDF-1α, eotaxin, BDNF and PDGF-BB have high factor loadings (0.675, 0.582, 0.679 and 0.72, respectively). Component 2, explaining 16.17% of the total variance, is closely associated with inflammation as the cytokines TNFα, MCP1 and eotaxin have high factor loadings (0.859, 0.806 and 0.439, respectively). Increased IGF-1 (PDGF-BB is weakly associated) loads very highly on component 3 (0.914), which explains 13.12% of the total variance and can be associated with growth. Component 4, explaining 11.94% of the total variance, can be interpreted as adiposity as leptin and TG/HDL have high factor loadings (0.744 and 0.745, respectively). According to this model, growth and inflammation are determined by three distinct factors that link together (Fig. 1q). However, adiposity could be determined by an independent factor.

Chemokine and growth factors as predictors for insulin resistance in prepubertal patients with obesity

After assessing binary logistic regression (n = 120; Table S5) and adjusting for the best combination of predictors (backwards to step 6), the results showed that leptin, TG/HDL, IGF-1, SDF-1α, TNFα, MCP1, BDNF and PDGF-BB were the strongest biomarkers for IR. The overall success rate (percentage of correct predictions) was 89.6. As determined by the analysis of the areas under ROC curves from the combined set of these eight variables, the sensitivity and specificity were 93.2% (ROC–AUC = 0.932, 95% confidence interval: 0.885–0.979; Fig. 1r).

Regarding the factor analysis, we also performed an ROC curve analysis from those variables with higher factor loadings. The best combined set of variables was leptin, TG/HDL, IGF-1, TNFα, MCP1 and PDGF-BB. The analysis indicated a sensitivity and specificity of 93.2% (ROC–AUC = 0.932, 95% confidence interval: 0.884–0.979; Fig. 1s). This result indicates that the six variables together highly contribute to a reliable model predicting IR.

Discussion

Here we investigated the variations of circulating pro-inflammatory and anti-inflammatory cytokines (TNFα, IL1β, IL4, IL6 and IL10), chemokines (SDF-1α, MCP1, eotaxin and fractalkine) and growth factors (BDNF, PDGF-BB and IGF-1) in IR of children with obesity and explored the combination of these molecules as a diagnostic tool in clinical practice. The data analysis indicates that some of these molecules are reliable biomarkers of the presence of IR. The pathophysiological relevance is reinforced by the association of IR conditions with differential circulating concentrations of MCP1, eotaxin and PDGF-BB in serum. We defined two predictive models that include the combination of leptin, TG/HDL, IGF-1, TNFα, MCP1 and PDGF-BB with an optimal sensitivity and specificity of 93.2%. We suggest that the combination of these circulating parameters from a single fasting sample could be useful to predict IR in childhood obesity.

An important novel contribution of the present study is the demonstration of an association between IR and the chemokines MCP1 and eotaxin, as well the growth factor PDGF-BB. Decreased circulating levels of MCP1 and eotaxin and increased circulating levels of PDGF-BB were able to differentiate IR and non-IR prepubertal children with obesity and suggest that IR underlies a derangement associated with inflammatory chemoattraction and growth. Additional pro-inflammatory (TNFα), chemokine (SDF-1α) and growth (BDNF and IGF-1) factors that comprise three linked components, as well as leptin and TG/HDL (as a fourth unlinked component), together contribute to determine the variance (65.9%) that explains IR.

Obesity-mediated IR leads to adipose tissue inflammation and the accumulation of pro-inflammatory macrophages, which are most likely the cells

expressing the pro-inflammatory cytokines TNFα and IL1β (24). It has also been suggested that inflammation may even be a mechanism to counter IR, as inhibition of adipose tissue inflammation results in glucose intolerance (25). In humans, increased BMI is associated with macrophages expressing TNFα in white adipose tissue (26), and IR is correlated with elevated MCP1 levels in visceral adipose tissue of subjects with obesity (27). IR is also associated with MCP1-mediated macrophage accumulation in skeletal muscle in mice and human (9). We found an increase in circulating levels of TNFα and MCP1 compared with controls, with a highly positive bivariate correlation between these two factors. However, no differences in TNFα and lower MCP1 levels were observed in children with obesity and IR when they were compared with non-IR subjects with obesity. This observation suggests a reduction in the recruitment of circulating monocytes and of the IR effect on macrophage activation in the context of prepubertal obesity.

Serum levels of MCP1 and eotaxin are increased in adult subjects with metabolic syndrome and positively correlated with macrophage-specific CD68 expression in adipose tissue of patients with obesity (11,28). Interestingly, treatment with the lipid-lowering agent atorvastatin in a randomized controlled trial reduced circulating levels of eotaxin and mRNA expression of its chemokine receptor CCR5 in peripheral CD14(+) monocytes (28). Levels of MCP1 and eotaxin were also found to be dominantly expressed in preadipocytes compared with adipocytes, suggesting a role of these chemokines in adipogenesis (29). In our study, there was a positive correlation between the circulating levels of MCP1 and eotaxin; however, while no change in circulating levels of eotaxin was observed in children with obesity, a significant decrease in eotaxin levels was found in the IR patients.

Stromal cell derived factor 1α is another adipocyte-derived chemotactic factor involved in obesity-induced adipose tissue inflammation and systemic IR (8). Several authors have questioned the role of SDF-1α as a mechanism of obesity-associated infiltration of macrophages into white adipose tissue (30). Here, an implication of SDF-1α was not found when it was considered alone in IR children; however, there was a relevant contribution of SDF-1α in component 1, a main component linking inflammation and growth with IR, when its variance was added to those of MCP1, eotaxin, PDGF-BB and BDNF.

The pro-fibrotic growth factor PDGF-BB and its receptor β play a key role in the development of adipose tissue vascularization and appear to be a key target for the prevention of obesity and T2DM (31). Indeed, M1 macrophages are a major cell type expressing PDGF-BB in obese adipose tissue (32). In patients affected by metabolic syndrome or cardiovascular events in T2DM, circulating levels of PDGF-BB are decreased (15). Our observations in children with obesity confirm a reduction in circulating PDGF-BB levels and, in turn, a likely reduced permeability of vascularity. However, we observed an increase in circulating levels of PDGF-BB in IR compared with non-IR boys with obesity, which suggests that PDGF-BB may act on vascular permeability to compensate for insulin desensitization in target tissues, as well as to increase macrophage infiltration (33). We also detected a possible sexual dimorphism in PDGF-BB, given that boys with obesity had increased levels compared with girls with obesity in an IR-independent manner. Although there are clear associations between specific cytokines/chemokines and obesity-associated IR, other factors could have important influences on these associations, such as diet (34) and other associated pathologies (35). Indeed, plasminogen activator inhibitor-1 has recently been shown to associate not only with IR but also with cardiovascular risk and the severity of liver affectation in children with non-alcoholic fatty liver disease (35).

In conclusion, the present study indicates that the combination of leptin, TG/HDL, IGF-1, TNFα, MCP1 and PDGF-BB can be useful as a potent diagnostic tool in clinical practice to predict IR in prepubertal obesity. Specifically, significant changes in circulating levels of TNFα, MCP1 and PDGF-BB were highly associated with IR in children with obesity. This fact suggests that TNFα, acting as pro-inflammatory factor in an obesity context, and PDGF-BB, as a vascular/fibrotic factor in an IR context, may differentially alter IR-associated MCP1 action and macrophage chemoattraction before puberty.

Conflict of interest statement

The authors have nothing to disclose.

Author contribution

P. R., G. A. M.-M., V. B., J. S., F. J. P., J. A. C., F. R. F. and J. A. were responsible for the study concept and design. P. R., G. A. M.-M., V. B., J. A. C and J. A. contributed to the acquisition of data. P. R., G. A. M.-M., V. B., J. S., F. J. P., J. A. C., F. R. F. and J. A. assisted with data analysis and interpretation of findings. P. R., G. A. M. M., V. B. and J. S. drafted of the manuscript. F. R. F. and J. A. provided critical revision of the manuscript for important intellectual content, obtained funding and study supervision. All authors approved final version for publication.

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Conflict of interest statement

No conflict of interest was declared.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Plasma concentrations of cytokines in prepubertal patients with obesity and controls.

Table S2. Plasma concentrations of cytokines in prepubertal patients with obesity regarding IR and sex differences.

Table S3. Relationships between chemokines and growth factors in prepubertal patients with obesity and IR or without IR (n = 120).[a]

Table S4. Chemokines and growth factors with high loadings in extracted components that contribute towards IR in prepubertal patients with obesity (n = 120).[a]

Table S5. Chemokines and growth factors as predictors for IR in prepubertal patients with obesity (n = 120).[a]